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ESTABLISHMENT OF A MICROSPORE CULTURE PROGRAM TO PRODUCE DOUBLED-HAPLOID LINES OF PEA AND BARLEY

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Establishment of a microspore culture program to produce doubled-haploid lines of barley and pea.

Final report on ADF project #98000067

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A. ABSTRACT

This project investigated the application of haploid technology to barley and field pea using isolated microspore culture. Published protocols from barley were adapted to local genotypes and facilities. Improvements to the procedure resulted in production of doubled-haploid barley lines from most crosses with reduced input costs. No protocol for doubled-haploid technology in pea existed. To date, a number of factors have been identified that allow development of haploid pea embryos from several genotypes. For the first time, a doubled-haploid plant was regenerated directly from a microspore. Efforts continue to identify key factors to develop routine doubled-haploid technology for pea.

B. EXECUTIVE SUMMARY

This project involved the application of isolated microspore culture to barley and field pea to develop doubled-haploid lines for the CDC breeding programs.

Published barley protocols were adapted to local genotypes and facilities. Adjustments were made and efforts continued to streamline the system to make it more cost efficient. An improved procedure is now in place that results in the routine production of 150-200 doubled-haploid plants from each of a wide variety of barley crosses, with six crosses processed every three months. Throughout the project, 3912 doubled-haploids were developed from 71 crosses. This technology helps speed up the development of new barley cultivars with improved traits.

In contrast to barley, no protocol for pea doubled-haploid technology exists. A large number of factors affecting microspore culture were investigated including donor plant conditions, genotype, pre-treatments, developmental stage of pollen grains, medium components, culture environment, and regeneration protocols. For the first time, a doubled-haploid plant from the cultivar Highlight was regenerated directly from a microspore and seeds were obtained. However, this technology needs to be applicable to a wide range of genotypes to be useful to breeding programs.

Doubled-haploid technology for pea would simplify the use of molecular markers for improvement of disease resistance as well as speed the release of resistant cultivars by at least two years resulting in considerable savings to the breeder. For the grower, improved cultivars would be available more quickly and thus reduce input costs for pea production. Development of this technology is still in its early stages and will require additional collaborative research to obtain a protocol that is as widely applicable and efficient as it is now for barley.

C. TECHNICAL REPORT

1. Project Background

Microspores (see section G Figs. 6, 20, 21) are immature pollen grains with a haploid chromosome set. Any plants developed from microspores are consequently haploid and upon doubling, either spontaneously or by induction, are completely homozygous. Some advantages of this system are: reduction in the time required for the development of new cultivars, potentially more efficient selection for specific traits, easier identification of recessive genes, and aiding molecular-marker assisted selection.

Microspore culture systems have been developed for barley and are already in use in European, USA, Australian, and other Canadian breeding programs. However, this procedure needs to be adapted to local genotypes as well as to local facilities. In addition, staff require training in the application of this complex technology.

Regarding pea, no such technology exists, thus, a large number of factors affecting microspore culture response have to be investigated including donor plant conditions, genotype, pre-treatments, developmental stage of the pollen grain, medium components, culture environment, and regeneration protocols.

2. Project Objectives

2.1. Barley

- To adapt published protocols for barley microspore culture to local breeding material.
- To train staff in the application of microspore culture.
- To provide 150 to 200 doubled-haploid lines from selected crosses to the CDC feed barley breeding program.
- To make this system more cost efficient.

2.2. Field Pea

- To develop a microspore culture system for pea in order to produce doubled-haploid lines for the CDC Pulse breeding program. Specifically:
  i. Identification of responsive genotypes
  ii. Evaluation of factors influencing microspore culture response.

(a) Donor plant conditions
(b) Pre-treatments
(c) Determination of the responsive pollen grain stage
(d) Composition of the culture medium
(e) Culture environment

iii. Evaluation of factors influencing regeneration
iv. Chromosome-doubling techniques
v. Doubled-haploid plants
vi. Technology application

3. Results and Discussion

3.1. Barley (Hordeum vulgare L.)

The microspore culture protocol used in this project was developed by Dr. Ken Kasha and co-workers from the University of Guelph (Kasha et al. 1997a,b).

3.1.1. Crosses processed from the 1998 crossing program

An initial assessment of this protocol was conducted using material from crosses made during 1998 in the CDC barley breeding program. Results are presented in Table 1. The number of doubled-haploid plants obtained from 19 crosses varied considerably (0 to 118). This was caused by substantial losses due to high contamination rates in culture, a low ratio of green to albino plants, and large numbers of sterile (haploid) plants. Steps were taken to address the inefficiency of this system.

Contamination resulted from environmental factors, insufficient sterile techniques, and donor plants. Regarding environmental factors, the laboratory was cleaned, laminar flow benches were inspected and leaks repaired, and an air filter was purchased to reduce the amount of mold in the air. Regarding sterile technique, staff received additional training and more supervision. However, sterilization of the plant material was a major challenge. At that point, only a surface sterilization with 70% ethanol was conducted which was insufficient for many genotypes. Hence, the antibiotic cefotaxime was added to the medium at a rate of 100 mg/L (Davies and Morton 1998).

The low ratio of green to albino plants and the large number of sterile plants were (in part) caused by sub-optimal donor plant growth conditions especially due to large variations in moisture levels over the course of one day. Barley does not tolerate too wet or too dry conditions. To minimize this effect, donor plants were grown in larger pots (Fig. 1) to reduce variation in soil moisture content. Furthermore, improvements were made to the fertilizer regime (60g/pot Osmocote® and 4g/pot Nutritrace®) and the growing temperature regime (22°C

day and 18°C night with a 12 hour day/night cycle during the germination and tillering phase).

Table 1. Barley crosses processed from the 1998 CDC crossing program.

MC #	Cross	No. of DH Plants
9836	HB351 x SB96674	41
9829	HB350 x SB96002	118
9837	HB348 x SB96473	5
9831	HB351 x TR253	39
9851	TR348 x TR255	98
9831	HvPal-1 x CDC McGwire	45
9810	SB90300 x CDC McGwire	31
	926K2#1 x McGwire	22
	HB348 x TR345	4
	Lysimax x CDC Freedom	10
	RFLP Harr. #3 x 926K2#1	12
	SB96002 x TR344	10
	TR253 x Prosa	0
	TR255 x 91A6991	24
	TR333#3 x 926K2#1	6
	TR640 x McGwire	23
	SH97119 x TR255	0
	C061	35
	WC009	5
	Total	528

3.1.2. Crosses processed from the 1999 crossing program

Improvements to the protocol were then applied to material from the 1999 CDC crossing program. Results are presented in Table 2. The number of doubled-haploid plants obtained from each cross still varied considerably (1 to 184). Furthermore, the system was still inefficient due to a low ratio of green to albino and fertile to sterile plants, as well as high contamination rates. Donor plants remained a source of contamination. Normally, spikes enclosed in the leaf sheath (see Fig. 2) were collected when the distance between the flag leaf and the penultimate leaf was 3 to 6 cm (i.e. microspores were in the mono-nucleate stage). However, in some crosses, the required distance was less than 1 cm and awns were clearly visible (see Fig. 3.) thus compromising the protection of the leaf sheath. Hence, in addition to the ethanol surface sterilization step, spikes were treated with a 15% bleach solution for 15 minutes. Humidity levels in the

growth chambers were also lowered to about 50% in order to reduce the mold level.

Table 2. Barley crosses processed from the 1999 CDC crossing program.

MC No.	Cross	No. of DH Plants
9912	HB357 x M95	44
9918	HB347 x TR258	86
9920	HB348 x TR253	52
9924	HB353 x Gairdner	25
9925	HB353 x Moravian	13
9928	TR253 x Xena	14
9929	TR253 x CDC Helgason	25
9934	TR258 x TR347	88
9939	TR347 x Gairdner	35
9940	TR347 x BM9203-74	10
9941	TR349 x TR353	49
9945	SH98595 x TR253	12
9946	SH98595 x HB347	27
9950	HDE84194 x BM98	42
9951	HDE84194 x HB353	22
9952	SH98382 x HB348	5
9953	SH98382 x BM9203	1
9954	SB98306 x TR258	184
9960	LP-H422 x HB357	22
9961	LP-h422 x HB347	24
9962	LP-h422 x TR253	29
9963	MC9813 x CDC McGwire	5
9964	M2-635 x 3*CDC Freedom	1
9965	Hvpa2 x 3*HB350	3
9966	Hvpa1 x 3*CDCMcGwire	1
9967	Valier x TR346	6
	Total	825

3.1.3. Manley experiments

Recent publications (Caredda and Clement 1999, Carreda *et al* 1999; Castillo *et al* 2000; Kasha *et al* 2001a,b; Mityko *et al* 2000) reported improvements to the microspore culture procedure. Several recommendations were tested using the cultivar Manley.

Test 1 involved two different spike stress pre-treatments (4 days at 4°C in 0.3M mannitol compared to 28 days at 4°C). Results showed that the first treatment was slightly better and, since it is considerably faster, this pre-treatment was adopted.

Test 2 compared separation of viable from non-viable microspores using maltose gradient centrifugation versus no separation (current method). There were no differences between the two treatments, however, if microspores from one cross were particularly poor, this procedure might be worth trying.

Test 3 involved the determination and adjustment of microspore density to 5 x 10^5 microspores per ml. This density was appropriate for liquid medium but too low for solid medium.

Test 4 compared solid (Kasha et al. 2001 a, b) versus liquid induction (Davies and Morton 1998) media. Liquid medium for 3 weeks was clearly detrimental to embryo development. Embryos developed faster on solid medium with fewer albino plantlets.

Test 5 compared a photoperiod 'duplicating' growth conditions in the field to a constant 16-hour day/ 8-hour light photoperiod. There appeared to be little difference between the two treatments. The donor plants in the 'field conditions' growth chamber tended to tiller less.

Test 6 investigated light quality, with three comparisons:

(1) greenhouse conditions with a light intensity (on average) of 350 $\mu Es^{-1}m^{-2}$
(2) high pressure sodium vapour lights in environmental chambers with a light intensity of approximately 450 $\mu Es^{-1}m^{-2}$
(3) double rows of fluorescent lights in environmental chambers with a light intensity of approximately 400 $\mu Es^{-1}m^{-2}$.

Greenhouse-grown donor plants were clearly inferior due to low light conditions (fall/winter) even with additional illumination. Greenhouse-grown material was also exposed to pests (e.g. thrips) and diseases (e.g. mildew) which posed problems. Temperature control was not an option (best growth conditions are 18° C day and 15° C night) and outside temperatures of -20°C and lower made transfer of plant material from the greenhouse (not in-house) to the lab difficult. Plants grown under fluorescent lights had better colour but weaker stems than those grown under high pressure sodium lights. Regarding microspore quality, there was no difference, however, a 3-4 week initial growth period under high pressure sodium lights may be beneficial for chlorophyll development. Hence, even though environmental chambers are more expensive, they provided the best growth conditions for donor plants.

Test 7 investigated the application of low levels of colchicine during the first two days of the induction period (Mityko *et al* 2000). Results have been ambiguous. Some crosses did not respond to colchicine treatment, others did. This could be due to genotypic differences between crosses. Poor responding crosses (often have a malting barley component) might benefit more from an application of colchicine.

In conclusion, the method developed by Kasha *et al* (2001a,b) was still the best system for Canadian genotypes.

3.1.4. Crosses processed from the 2000 crossing program

Results from these tests were applied to crosses MC0040, MC0059, MC0067, MC0068A, MC0069A, MC0070A, and MC0071A from the 2000 CDC crossing program (Table 3). Since donor plant growth was identified as one of the most critical steps in the microspore procedure, an automatic watering system was tested on these crosses as well. It was found ineffective because even water distribution could not be achieved (i.e. plants at the beginning of the line received too much water, plants at the end of the line did not receive enough). The system was removed as soon as it was determined that there was no technical solution to this problem. However, by this time the quality of most donor plants was already adversely affected resulting in low numbers or doubled-haploid plants with the exception of cross MC0040. Barley plants were sensitive to over-watering during the early stages and under-watering during the later stages of development. Poor quality donor plants lead to poor quality microspores and once donor plants were negatively affected, nothing can be done to reverse this effect.

In May of 2001, Dr. Ken Kasha visited our laboratory and recommended applying the following changes to our microspore culture protocol:

1. Using less mannitol (12ml) during cold pre-treatment.
2. Adding 10% FHG (Kasha *et al* 1997a,b) nutrients to mannitol.
3. Shorter blending times (about 5 seconds).
4. Frequent changes of 100 µm nylon filters.
5. Spreading developing embryos over the entire FHG plate in order to ensure good contact with the medium and prevent starvation.
6. Adding 0.1g/L gibberellic acid to the liquid FHG medium.
7. Reducing the concentration of phytagel from 3 g/L to 2.75 or 2.5 g/L.
8. Earlier transfer of embryos (about 2 mm) to MS medium.

These recommendations were tested on crosses from the 2000 CDC crossing program (Table 3) with the exception of the 7 crosses mentioned above. The response to the improved protocol was excellent and large numbers of doubled-haploid plants were obtained. Since growth chamber and greenhouse space as

well as manpower was limited, material from several crosses was discarded (+) after consultation with the breeder in charge of the feed barley program.

Table 3. Barley crosses processed from the 2000 CDC crossing program.

MC #	Cross	No. of DH Plants
0040	CI4196 x CDC Helgason	113
0052	Valier x TR 253	284+
0059	CI4196 x CDC McGwire	33
0061	LP955 x SH 98326	26+
0067	MC9813-11/CDCMcGwire x CDC McGwire	16
0068A	Haruna nijo #1 x RFLP Harrington #1	5
0068B	Haruna nijo #2 x RFLP Harrington #1	144+
0068C	Haruna nijo #3 x RFLP Harrington #1	217+
0068D	Haruna nijo #5 x RFLP Harrington #2	0+
0069A	Haruna nijo #1 x TR253 #2	0
0069B	Haruna nijo #2 x TR253 #3	307+
0069C	Haruna nijo #4 x TR253 #3	78+
0069D	Haruna nijo#6 x TR253 #2	8+
0070A	Haruna nijo #3 x SM94291 #2	0
0070B	Haruna nijo #4 x SM94291#2	14+
0070C	Haruna nijo #5 x SM94291 #1	2+
0070D	Haruna nijo #5 x SM94291 #2	0+
0070E	Haruna nijo #6 x SM94291 #1	357+
0071A	Haruna nijo #1 x CDC Kendall #1	2
0071B	Haruna nijo #5 x CDC Kendall #1	154+
0071C	Haruna nijo #6 x CDC Kendall #2	0+
	Total	1760

+ Indicates that large numbers of green plantlets were produced but the plants were not advanced due to lack of greenhouse space.

Since the protocol was working well, efforts began to streamline the procedure in order to make it more cost efficient and less labour intensive using the material from 5 more crosses from the 2000 CDC crossing program (Table 4). The following changes were tested:

1. Reducing the number of donor plants from 5 to 4.
2. Reducing the time of spike collection (from 4 to 2 weeks).
3. Reducing the number of extractions and FHG plates (Fig. 4) per cross.
4. Instead of transferring plantlets from MS plates (Fig.13) to Magenta boxes (Fig. 14), plantlets were transferred directly to Jiffy® pots (Fig.15).

5. Instead of growing each plant in a separate pot, 6 plantlets were planted in one large pot (Fig. 16) reducing the time required for watering, labeling, and saving greenhouse space.

Table 4. Barley crosses from the 2000 CDC crossing program currently being harvested.

MC #	Cross	No. of DH Plants
0032	TR 359 x TR 256	150 +
0036	SB 98318 x TR 256	210
0049	SH 99707 x HB 352	219 +
0051	Valier x CDC Bold	20 +
0060	LP 955 x HB 358	200 +
	Total	799

+ Indicates that more green plants were produced but not further processed.

Results showed (Table 4) that with this streamlined protocol, 150-200 doubled-haploid lines could be produced from each cross and thus our objective was met.

3.1.5. Crosses currently being processed

Using material from the 2001 crossing program (Table 5 and 6) the following improvements will be tested:

1. Increase the number of crosses from 5 to 6 per set (every three months). This allows optimal use of our growth chamber facilities.
2. Increase the number of plantlets per pot from 6 to 8 to decrease the amount greenhouse space required.

Table 5. Barley crosses from the 2001 CDC crossing program, with doubled-haploid seed currently being harvested.

MC No.	Cross	No. of Plants in Soil
0181	H00752 x 9-1LS x CS-CDC McGwire	50 +
0110	SH99119 x BT 474	50 +
0112	SH99119 x 98236	285 +
0134	TR 263 x TR 710	145
0154	HDE 84194-622-1 x CDC Helgason	345

+ Indicates that more green plants were produced but not further processed.

Regarding the first five crosses (Table 5), plants are currently being transferred to soil. Regarding the second set (Table 6) from the 2001 crossing program, green plantlets are currently being transferred to MS plates and to Jiffy® pots. Resulting doubled-haploids will be available in the fall of 2002 for seed increase in the 2002/03 New Zealand winter nursery.

Table 6. Barley crosses from the 2001 CDC crossing program currently being processed.

MC #	Cross	No. of FHG Plates
BM9935	TR251 x CDC Bold	7
0119	SH97013 x 99-509-01-090	9
0139	TR710 x TR366	6
0156	HDE84194-622-1 x CDC McGwire	6
0160	SB99158 x TR359	11
0170	SH00749 x CDC McGwire	5

Seeds from barley crosses from the 2001-2002 crossing program (Table 7) have been planted in May of 2002 and spike collection will start at the end of June.

Table 7. Barley crosses from the 2001-2002 crossing program.

MC #	Cross	No. of Donor Plants
0133	TR361 x TR263	4
0135	TR710 x CDC Helgason	4
0159	TR361 x 2 ND16092	4
0173	2 nd 16092 x HB361	4
0201	AC Ranger x SB97590	5
0215	HB373 x CDC McGwire	4

3.1.6. Conclusions and Further Development

The objectives of the barley sub-project have been accomplished. Published protocols for barley microspore culture were adapted to local breeding material and facilities. Staff were trained in the application of this method. 150 to 200 doubled-haploid lines are now routinely produced per cross for the CDC feed barley breeding program. Efforts were made to streamline this procedure (for improved protocol see: F. Project Developed Materials) and costs and labour have been reduced considerably. Our routine procedure will see 150-200 doubled-haploids produced from each of six crosses every three months. Doubled-haploid seed increases will be undertaken twice annually, once at Saskatoon and once in our annual New Zealand contra-season nursery. Barley

doubled-haploid production using microspore culture can now be incorporated into the CDC feed and malting barley programs.

3.2. Field Pea (*Pisum sativum* L.)

Androgenesis is a multi-step process that requires each part to function in order to yield doubled-haploid plants. Evaluation of any of the factors cannot be done in isolation since they are inter-connected. For example, identification of responsive genotypes requires a medium that allows embryo development from microspores. However, developing a medium for embryo regeneration requires responsive genotypes. Furthermore, if (for example) the microspore density is sub-optimal or if donor plant growth is poor (see barley), embryo development might be completely inhibited even though the genotype might be responsive and the medium might be optimal. Hence, even though one factor might have been determined once, whenever any of the conditions changed, this factor might need to be re-evaluated under the new conditions. Developing a predictable and repeatable microspore culture protocol for field pea is laborious and slow. This project represents the beginning stages of developing a repeatable protocol for androgenesis in field pea.

3.2.1. Identification of Responsive Genotypes

Androgenetic response was shown to be genotype specific for both embryo induction and plantlet regeneration in many crops (for a review see e.g. Ferrie *et al* 1995). Hence, an experiment was conducted to identify responding genotypes using 2,4-D alone or in a combination with a heat (37°C) pre-treatment of 6 hours and using an L6-based medium (Kumar *et al* 1988).

Thirty field and sixteen garden pea cultivars were evaluated and results are presented in Table 8. Highlight, CDC April, CDC Mozart and Blizzard were the most responsive cultivars under the conditions tested and used in subsequent experiments. However, genotype by pre-treatment interactions were observed (for details see section 3.2.2.b).

3.2.2. Evaluation of Factors Influencing Microspore Culture Response

(a) Donor plant conditions

As mentioned above for barley, donor plant growth has a major impact on androgenesis. Hence, optimal growing conditions were important for obtaining

responsive microspores. Donor plants were grown in 30cm plastic pots using Redi-earth®, with 5-6 plants per pot. Two weeks after seeding, the plants were

Table 8. Pea cultivar screening

Field Pea Cultivar	Response	Garden Pea Cultivar	Response
AC Melfort	Poor	Dark Skin Perfection	o.k.
Alfetta	Poor	Freezer 69	Poor
Baccara	Poor	Green Arrow	Poor
Badminton	Poor	Greenfeast	Poor
Bohatyr	Poor	Homesteader	Poor
Blizzard	Good	Laxton's Progress	Poor
Carnaval	Poor	Little Marvel	Poor
Carrera	Poor	Novella	Poor
CDC Allegro	Poor	Oregon Giant	Poor
CDC April	Good	Oregon Sugar Pod	Poor
CDC Handel	o.k.	Parsley Prairie	Poor
CDC Minuet	Poor	Puget	Poor
CDC Mozart	Good	Scout	Poor
CDC Peko	Poor	Spring	Poor
CDC Sonata	Poor	Strategem	Poor
CDC Verdi	o.k.	Tall Telephone	Poor
CDC Vienna	Poor		
CDC Winfield	Poor		
Cobra	Poor		
Cresta	Poor		
Croma	Poor		
Delta	Poor		
Eclipse	Poor		
Grande	Poor		
Greenfeast	Poor		
Highlight	Very Good		
Kazar	o.k.		
Keoma	Poor		
Orb	o.k.		
Radley	Poor		

Poor = no globular embryos
o.k.= one to 5 globular embryos per plate
Good = 5 –10 globular embryos per plate
very good = more than 10 globular embryos per plate

fertilized with 75 g per pot of Nutricote® 13-13-13 N:P:K slow release fertilizer together with 3 g per pot of 20-20-20 N:P:K water soluble fertilizer. In addition, flowering plants were given 2 g per pot of the water-soluble flowering fertilizer once per week.

Preliminary experiments growing donor plants in the greenhouse versus growth chamber conditions was conducted for several pea cultivars. Results showed that similar to barley, plants grown in the greenhouse had problems with pests and diseases. Hence, subsequent experiments used only material grown in growth chambers under controlled conditions.

A comparison of influorescent/ incandescent (app. 245 $\mu Es^{-1}m^{-2}$) and high sodium (app. 450 $\mu Es^{-1}m^{-2}$) light conditions showed no clear difference between the two light sources. However, this could have been due to the low embryogenic response in this experiment. This factor might need to be re-evaluated when the protocol has been further optimized.

A number of experiments were conducted with various temperature regimes. Results showed that reducing the temperature from a 22° C day/16°C night regime to 18°C day/13°C night during bud formation improved microspore quality and also prolonged the time for bud collection. In general, plants are now grown under incandescent and fluorescent lights with a two-phase temperature regime of 22°C day (16 hours) and 18°C night (8 hours) until they are close to bud formation (to shorten the juvenile period), followed by 18°C day and 13°C night during bud collection. Humidity could not be controlled in the growth chamber available at that time. However, recent experiments in humidity-controlled growth chambers indicated that a 50% humidity level reduced the occurrence of mold contamination in pea cultures similar to barley.

(b) Pre-Treatments

Using three pea cultivars (Blizzard, CDC April and CDC Mozart) the effect of cold (4°C) and heat (37°) stress was tested for periods of 1, 2, 4, 6,12, 24, 48, and 96 hours. Microspores were kept for 3 days in liquid culture after the application of the stress treatment and were then evaluated using DAPI staining. Results showed that 1 to 6 hours of heat treatment or 96 hours of cold treatment were necessary in order to observe symmetrical microspore nuclei divisions (Fig. 23-25) with heat being effective faster than cold. Both heat and cold treatment were effective for cultivars CDC April and Blizzard but were less effective for cultivar CDC Mozart. Pre-treatment conditions might have to be re-investigated for the latter cultivar.

Another pre-treatment experiment involved placing buds for three to ten days in solutions of different (0.3, 0.4, 0.5 and 0.6 M) concentrations of mannitol. This treatment did not improve induction. The next test combined the effect of cold with 0.3M mannitol. This resulted in a positive effect for cultivar CDC April depending on the medium used but negatively affected cultivar Blizzard.

(c) Developmental stage of the pollen grain and embryo development

Pea buds contained pollen in different stages of development from tetrad (Fig. 18), just after release from tetrad (Fig. 19), mono-nucleate (Fig. 20 and 21) and bi-nucleate (Fig. 22) with a dense generative and a diffuse vegetative nucleus. Mono- and bi-nucleate microspores were oval and measured about 80 micron in length and about 45 micron in width.

Bud size varied considerably with each cultivar or genotype as well as over time. Generally, buds were chosen in which the majority of the microspores were in the mono-nucleate stage. At this time, the anther colour was green to yellowish.

After application of stress treatment and/or 2,4-D, a switch from the normal gametophytic (Fig. 22) to the sporophytic (Fig. 23) pathway was observed in some experiments. Microspore nuclei division was clearly symmetrical (Fig. 23 - 25). Embryo development followed the usual stages from pro-embryo (Fig. 26), through globular (Fig. 27), heart-shaped (Fig. 28) and cotyledonary stages (Fig. 29) and was obtained in a number of cultivars (see Table 8).

(d) Composition of the culture medium

The composition of the culture medium can have a major impact on androgenesis. Experiments were conducted to evaluate the different components of the culture medium. Specifically investigated were:

i. Base medium (Durand *et al* 1973, Gamborg *et al* 1968, Kaltchuk-Santos *et al* 1997, Kumar *et al* 1988, Lehminger-Mertens and Jacobsen 1989, Murashige and Skoog 1962, Veliky and Martin 1970, White 1983)
ii. Carbon sources (sucrose, fructose, glucose) and concentrations
iii. Various growth regulators (auxin, cytokinin) and concentrations
iv. Organic nitrogen supplements (amino acids and casein hydrolysate)
v. Osmotica (PEG, mannitol, sorbitol, melibiose and maltose).

Globular embryos were obtained using L6 medium (Kumar *et al* 1988) with 30g/L sucrose and 1 mg/L 2,4-D (Table 8). Some embryos developed to the heart-shaped (Fig. 28) or cotyledonary (Fig. 29) stage and in one case, an embryo from cultivar Highlight was regenerated into a plant (Fig. 30). However, globular embryos frequently callused indicating the persistent effect of 2,4-D that could not be removed regardless of subsequent treatments. Further tests showed that IAA might be a good substitute for 2,4-D.

(e) Culture environment

A number of experiments were conducted to determine the optimum culture environment regarding light intensities and incubation temperatures during the

induction period. Results indicated that temperatures below 25°C or above 27°C as well as exposure to light (high or low) did not improve induction. Hence, the best incubation conditions were 25°C in darkness until globular embryo development occurred (about 2 weeks) followed by 23°C and low light.

(f) Microspore density

Different microspore densities were also investigated. Results indicated that a density of 600,000 – 900,000 microspores per ml is required for liquid cultures in order to induce embryogenesis. However, induction on solid medium required a 'mound' of microspores similar to barley.

3.2.3. Evaluating factors influencing regeneration

Several embryo development media were tested with or without different combinations and concentrations of hormones (TDZ, BA, GA_3, ABA, NAA, zeatin), carbon sources and concentrations (sucrose, glucose, fructose, maltose), organic nitrogen sources, and pH. Since most globular embryos callused regardless of the medium used, these tests will have to be repeated with embryos derived from the IAA experiments. The microspore derived plant regenerated from cultivar Highlight was grown on MS medium without growth regulators.

At this point, only one plant could be regenerated directly from a microspore resulting from an experiment with 2,4-D in the induction medium. All attempts to root this plant failed and this plant was kept in Magenta boxes. El-Bahr *et al.* (1999) noted that rooting could not be induced in their somatic embryogenesis experiments if plants had been obtained by using 2,4-D. Consequently, this growth regulator needs to be replaced since grafting large numbers of plants is not an option in an efficient protocol.

3.2.4. Chromosome-doubling techniques

One plant was regenerated from cultivar Highlight (Fig. 30). Efforts were made to determine the ploidy level of this plant. This procedure was difficult due to the lack of root formation. Using vegetative growing points as source material, it was determined that more than 7 chromosomes were present, i.e. more than a haploid set. However, the exact number could not be ascertained. Since then, fully fertile seeds were obtained from this plant indicating that spontaneous chromosome doubling must have taken place (Fig. 31). It is possible that symmetrical nuclei division resulted in spontaneous fusion similar to barley.

3.2.5. Doubled-haploid plants

Similar to the barley system, seeds from doubled-haploid plants will be transferred to the CDC breeding program. Since this protocol is not yet applicable to a wide range of genotypes on a routine basis, no doubled-haploid plants other than the one obtained from cultivar Highlight were produced.

3.2.6. Technology Application

Once a routine protocol has been developed, it will be applied to crosses from the CDC pea breeding program for the improvement of traits such as mycosphaerella blight or lodging resistance.

3.2.7. Conclusions

1. Androgenesis using isolated microspore culture is also possible in pea.
2. The most responsive microspore stage is the mono-nucleate stage.
3. There are genotypic differences between cultivars as well as interactions between genotype and environmental factors.
4. The first indicator of androgenesis is the symmetrical microspore nuclei division similar to other crops.
5. The minimum density was found to be 600,000 to 900,000 microspores per ml for liquid cultures.
6. Embryos developed directly from microspores without an intervening callus phase.
7. Embryo development followed the usual stages from pro-embryo to globular and occasionally, heart-shaped and cotelydonary-shaped embryos.
8. The growth regulator 2,4-D is very effective for induction of embryos, however, its effect cannot be removed resulting in embryos that continue to callus as well as causing failure of rooting in regenerated pea plants.
9. At this point, the naturally occurring auxin indole acetic acid (IAA) seems to be an alternative for 2,4-D. Material obtained from the initial experiment has not shown any signs of callusing to date.

3.2.8. Further Developments

Androgenesis is a complex process that requires all steps to function at least to some extent in order to obtain embryo and plant development. To develop such a system is extremely laborious and time consuming because interactions between the different steps requires re-checking of all components if a change has been made to the protocol.

Current research involves re-testing basal medium composition, optimizing the microspore density at the time of plating as well as varying the IAA concentration in the induction medium. However, avoidance of the use of growth regulators in the induction phase would be a preferable solution and preliminary experiments indicate that auxins (e.g. 2,4-D or IAA) might not be required for androgenesis in pea. This could be accomplished by optimizing stress pre-treatments. Hence, experiments are underway to re-examine cold or heat stress, carbon starvation, carbon source and osmolarity of the medium, alone or in combination with each other.

Recently, Australia's Grain Research and Development Corporation has identified the development of haploid technology for pulses as one of their major research goals and has given preliminary funding approval for two 3-year projects involving pea. Efforts are underway to develop a collaboration between Australian and Canadian researchers. Considering the expenditures needed for this research and the length of time that was required for the development of this technology in other crops such as barley or canola, a joint project would be advantageous to both countries.

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D. Personnel

E. Equipment

No equipment was purchased with this grant.

F. Project Developed Materials

For posters see attached files or printouts.

List of barley crosses processed and number of doubled-haploid plants developed during this project:

MC #	Cross	No. of DH Plants
9836	HB351 x SB96674	41
9829	HB350 x SB96002	118
9837	HB348 x SB96473	5
9831	HB351 x TR253	39
9851	TR348 x TR255	98
9831	HvPal-1 x CDC McGwire	45
9810	SB90300 x CDC McGwire	31
-	926K2#1 x McGwire	22
-	HB348 x TR345	4
-	Lysimax x CDC Freedom	10
-	RFLP Harr. #3 x 926K2#1	12
-	SB96002 x TR344	10
-	TR253 x Prosa	0
-	TR255 x 91A6991	24
-	TR333#3 x 926K2#1	6
-	TR640 x McGwire	23
-	SH97119 x TR255	0
-	C061	35
-	WC009	5
9912	HB357 x M95	44
9918	HB347 x TR258	86
9920	HB348 x TR253	52
9924	HB353 x Gairdner	25
9925	HB353 x Moravian	13
9928	TR253 x Xena	14
9929	TR253 x CDC Helgason	25
9934	TR258 x TR347	88
9939	TR347 x Gairdner	35
9940	TR347 x BM9203-74	10
9941	TR349 x TR353	49
9945	SH98595 x TR253	12
9946	SH98595 x HB347	27
9950	HDE84194 x BM98	42
9951	HDE84194 x HB353	22
9952	SH98382 x HB348	5

9953	SH98382 x BM9203	1
9954	SB98306 x TR258	184
9960	LP-H422 x HB357	22
9961	LP-h422 x HB347	24
9962	LP-h422 x TR253	29
9963	MC9813 x CDC McGwire	5
9964	M2-635 x 3*CDC Freedom	1
9965	Hvpa2 x 3*HB350	3
9966	Hvpa1 x 3*CDCMcGwire	1
9967	Valier x TR346	6
0040	CI4196 x CDC Helgason	113
0052	Valier x TR 253	284 +
0059	CI4196 x CDC McGwire	33
0061	LP955 x SH 98326	26 +
0067	MC9813-11/CDCMcGwire x CDC McGwire	16
0068A	Haruna nijo #1 x RFLP Harrington #1	5
0068B	Haruna nijo #2 x RFLP Harrington #1	144 +
0068C	Haruna nijo #3 x RFLP Harrington #1	217 +
0068D	Haruna nijo #5 x RFLP Harrington #2	0 +
0069A	Haruna nijo #1 x TR253 #2	0
0069B	Haruna nijo #2 x TR253 #3	307 +
0069C	Haruna nijo #4 x TR253 #3	78 +
0069D	Haruna nijo#6 x TR253 #2	8 +
0070A	Haruna nijo #3 x SM94291 #2	0
0070B	Haruna nijo #4 x SM94291#2	14 +
0070C	Haruna nijo #5 x SM94291 #1	2 +
0070D	Haruna nijo #5 x SM94291 #2	0 +
0070E	Haruna nijo #6 x SM94291 #1	357 +
0071A	Haruna nijo #1 x CDC Kendall #1	2
0071B	Haruna nijo #5 x CDC Kendall #1	154 +
0071C	Haruna nijo #6 x CDC Kendall #2	0 +
0032	TR 359 x TR 256	150 +
0036	SB 98318 x TR 256	210 +
0049	SH 99707 x HB 352	219 +
0051	Valier x CDC Bold	20 +
0060	LP 955 x HB 358	200 +
71 crosses	Total number of DH plants produced	3,113

Furthermore, 17 other crosses are in various stages of development (see Tables 5-7).

The following protocol for isolated microspore culture was developed based on publications by Dr. Ken Kasha and coworkers (Kasha et al 1997, Kasha et al 2001a, b) as well as through experimentation with local material and growing conditions:

Barley Isolated Microspore Protocol

1. Donor Plant Growth (Fig. 1)

- Pots (20cm diameter and 20cm height) were prepared by filling them $^2/_3$ with Redi-earth®. In order to ensure even soil moisture, soil was watered from above and below using a diluted water-soluble 20-20-20 fertilizer.
- Pots were drained overnight to remove excess moisture.
- 60 g/pot Osmocote® and 4 g/pot Nutritrace® were added to each pot.
- A seed was planted about 1 cm deep in the centre of each pot.
- The entire soil surface was covered with about 3-4 cm of Turface ® .

2. Collection of tillers

- In order to induce microspores to switch from the gametophytic to the sporophytic pathway, the nuclei have to be in the uni-nucleate stage (Fig. 6). Hence, tillers were collected when the distance between the flag leaf and the penultimate leaf was about 3-6 cm (Fig. 2). However, this distance varied considerably between crosses (Fig. 3) as well as within crosses depending on the age of the donor plant. Hence, microspores were staged bi-weekly to ensure that the correct microspore stage was targeted.
- Cut tillers were kept in water until they could be processed.
- All subsequent procedures were carried out in a sterile laminar flow hood.

3. Sterilization procedure

- Each spike was remove from its surrounding leaf sheath and awns were nipped off (Fig. 4) .
- Spikes from one cross were collected in a sterile beaker containing a stirring bar.
- A 15% bleach solution was added and covered with a sterile top filter to keep spikes submerged.
- Spikes were then sterilize for 20 minutes on a stirring plate using a low speed in order to avoid damage to the spikes.
- The bleach solution was drained off and spikes were rinse thoroughly with sterile distilled water until all traces of bleach were removed.

4. Pre-treatment

- Spikes were transferred in one layer (not more than 10 to 15 spikes depending on size) to a deep Petrie® dish.
- About 20 ml sterile 0.3M mannitol + 10% FHG nutrients (no hormones) solution was added, just enough to cover the bottom of the plate.
- Each plate was wrapped twice with Glad® wrap.
- Each plate was labeled with coloured tape, MC#, and date.
- Plates were stored in the fridge at 4°C for 3-4 days.

5. Extraction procedure

- Spikes were cut into approximately 1cm pieces and put into blender cup.
- A small amount of sterile 0.3M mannitol was added.
- Spikes were blended for 5 seconds.
- The resultant slurry was strained through cheesecloth filter.
- This procedure was repeated once.
- The filtrate was then poured through 100 µm nylon filter.
- Filtrate was transferred to 15 or 50 ml centrifuge tubes.
- Centrifugation was carried out for 3 minutes at 1000 RPM in Centra® centrifuge using a swinging bucket rotor.
- The supernatant was carefully removed, fresh mannitol added, and the procedure was repeated.
- Microspores were checked at this point for contamination using a microscope.
- In case of bacterial contamination, the microspore solution was sieved using a 38-40 µm nylon mesh filter. The microspores were collected on top of the filter whereas the bacterial contamination passed through the filter and was discarded.
- The microspores were then washed off the filter with mannitol and re-centrifuged.
- The supernatant was again carefully removed and a small amount of FHG + PAA liquid medium added to dissolve the microspore pellet.
- A sterile porcelain funnel was attached to a vacuum flask.
- Two layers of sterile Whatman® #1 filter paper were put on a Buchner® funnel and the paper moistened with FHG +PAA liquid medium.
- The microspores were slowly pipetted onto the filter paper while using the vacuum. It was important to concentrate the microspores in one place (Fig. 5). If the microspore density was not high enough, the response was poor.
- The filter with the microspores was then transferred onto a plate containing solid FHG medium containing 100 mg/L cefotaxime.
- Plates were labeled with colour, MC#, and date.
- Each plated was twice wrapped with Glad® wrap.

6. Incubation

- Microspores were incubated at 25°C in the dark until embryos developed (Fig. 7-13).
- After about 2 weeks, 2-3 drops of liquid FHG (no hormones) +GA_3 medium was added depending on the dryness of the plate and in order to disperse embryos across the plate.

7. Transfer of embryos

- Embryos were taken from plates with FHG medium when they are about 2 mm in size and transferred to plates with MS differentiation medium (Fig. 14).
- Embryos were kept in an incubator without light for 3 days at 25°C and were then transferred to an incubator with low light intensity and 23°C temperature.
- Albino plantlets were removed from the plates to allow better growth of green plants.

8. Transfer of plantlets to soil

- When green plantlets were sufficiently developed, they were transferred to Jiffy® pots (Fig. 16).
- Jiffy® pots were prepared by soaking them in a dilute solution of 20-20-20 water-soluble fertilizer.
- A hole was made in center of the jiffy pot, plantlets were dipped into Stim-root #1® and put into hole. The soil was gently squeezed around the base of plantlets.
- The plantlets were then transferrd to clear plastic containers.
- Plantlets needed frequent spraying with water to keep them from drying out.
- Boxes were labeled with coloured tape and MC#.
- The boxes with the plantlets were kept in incubators or in growth chambers.
- Before transfer to pots, lids were opened for short periods in order to acclimatize plantlets to lower humidity.

9. Greenhouse

- Pots (18 cm height and 27 cm diameter) were filled with Redi-earth®, watered thoroughly and drained.
- One teaspoon of Osmocote® was added to each pot.
- The net surrounding the Jiffy® pots were removed and transferred to the pots.
- Each pot contained 8 plantlets of about the same size.
- Pots were labeled with coloured tape and MC#.
- The plantlets were covered with clear plastic cups if needed.

- After about one week, the cups were removed.
- The plants were then transferred to the greenhouse (Fig. 17).
- Plants were fertilized once a week with1 teaspoon of 20-20-20 per liter water-soluble fertilizer.

10. Harvest

- Spikes were harvested when they were dry and seeds were transferred to the CDC barley breeding program.

H. Acknowledgement

We acknowledge the financial support of the Saskatchewan Agriculture Development Fund for this project; the University of Saskatchewan for providing laboratory facilities and the Crop Development Centre, non-malting barley program for supplying crossed barley seeds to produce F_1 donor plants. We would also like to recognize the significant assistance provided to the barley project by Dr. K. Kasha, Professor Emeritus, University of Guelph, in form of guidance and assistance in transferring doubled-haploid technology from his laboratory to ours.

We also acknowledge the financial contribution by the Saskatchewan Pulse Growers and by the Canadian Adaptation for Rural Development Fund for this project. Furthermore, we recognized the financial contribution by the Canadian Foundation for Innovation to Dr. T. Warkentin for the purchase of laboratory equipment. The technical support and the seeds provided by the pulse breeding program are also acknowledged.

G. Project Photos: Barley

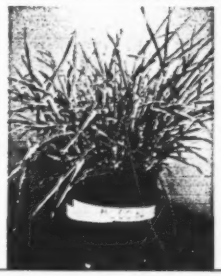

Fig. 1. Donor plant.

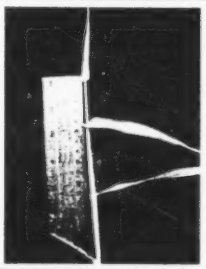

Fig. 2. Barley tiller.

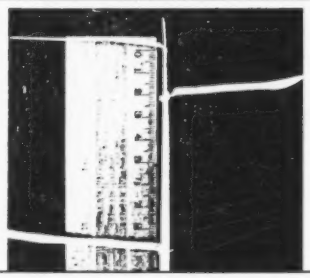

Fig. 3. Tiller with awns out.

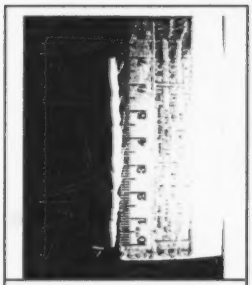

Fig. 4. Barley spike.

Fig. 5. FHG plate with mound of microspores.

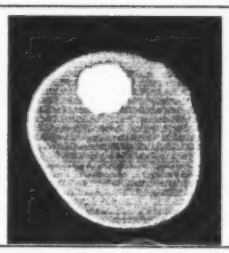

Fig. 6. Mono-nucleate microspore (DAPI stained).

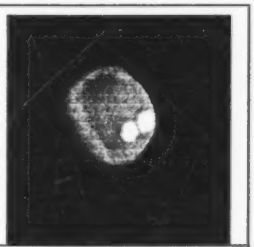

Fig. 7. Bi-nucleate microspore with two similar nuclei.

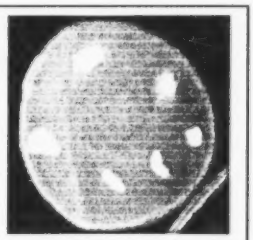

Fig. 8. Multi-nucleate microspore (DAPI stained)

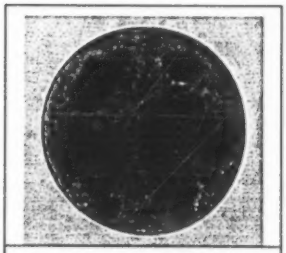

Fig. 9. Multi-nucleate microspore (aceto-carmine stained).

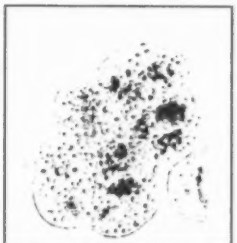

Fig. 10. Early multi-celled pro-embryo.

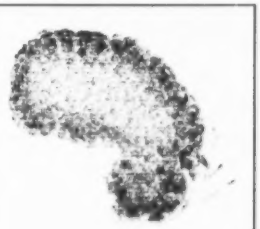

Fig. 11. Early embryo.

Fig. 12. Club-shaped embryo.

Fig. 13. FHG plate with embryos.

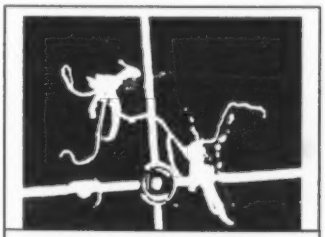

Fig. 14. MS plate with plantlets.

Fig. 15. Plantlets in Magenta boxes.

G. Project Photos: Barley - continued

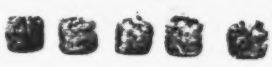

Fig.16. Plantlets in Jiffy® pots.

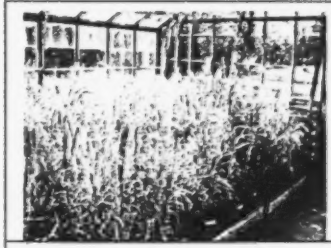

Fig.17. Doubled-haploid plants in the greenhouse.

G. Project Photos: Field Pea

Fig. 18. Tetrad with large central nucleus (acetocarmine stained).

Fig. 19. Microspore just after release from tetrad (acetocarmine stained).

Fig.20. Mono-nucleate microspore with central nucleus (acetocarmine stained.

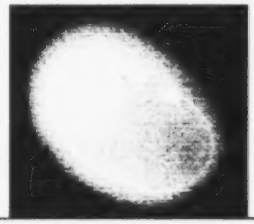

Fig. 21. Microspore with enlarged nucleus just prior to division (DAPI stained).

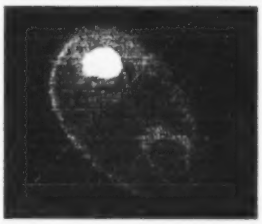

Fig. 22. Bi-nucleate microspore with dense generative and diffuse vegetative nucleus (DAPI stained).

Fig.23. Bi-nucleate microspore with 2 similar nuclei (DAPI stained).

Fig. 24. Microspore with four similar nuclei.

Fig. 25. Microspore with multiple nuclei (DAPI stained).

G. Project Photos: Field Pea - continued

Fig. 26. Early globular embryos with suspensors from cultivar CDC April (acetocarmine stained).

Fig. 27. Globular embryo on induction plate.

Fig. 28. Early heart-shaped embryo from cultivar CDC Mozart.

Fig. 29. Embryo from cultivar Blizzard.

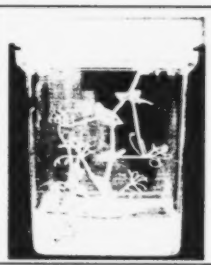

Fig. 30. Doubled-haploid plant from cultivar Highlight.

Fig. 31. Doubled-haploid pea plant.